MicroBioTest Protocol

AOAC Germicidal Spray Test
Supplemental

Mycoplasma orale

Testing Facility
MicroBioTest
Division of Microbac Laboratories, Inc.
105 Carpenter Drive
Sterling, VA 20164

Prepared for
Virox Technologies, Inc.
2770 Coventry Road
Oakville, Ontario
L6H 6R1

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MicroBioTest Protocol No.: 506.1.05.25.16

MicroBioTest Project No.: 506 - 153

OBJECTIVE:

This test is designed to prove germicidal effectiveness label claims for products registered with the Environmental Protection Agency and Canada (if applicable) as spray germicides. It evaluates the effectiveness of sprays and pressurized spray products as spot disinfectants for contaminated surfaces. The test is based on the Official Methods of Analysis (2012) and is required by EPA Product Performance Guidelines (OCSPP 810.2000 and 810.2200).

TESTING CONDITIONS:

Ten replicates will be evaluated using two lots of a single test substance. Glass carriers inoculated with *Mycoplasma orale* will be sprayed for the specified time and distance directed by the sponsor or label instructions and held for the exposure time and at the temperature specified by the sponsor. The carriers will be cultured, incubated and observed for visible growth.

MATERIALS:

A. Test, control and reference substances will be supplied by the sponsor of the study (see last page).

- The identity, strength, purity, and composition, or other characteristics which will appropriately define the test, control, or reference substance shall be determined for each batch and shall be documented by the sponsor before its use in a study. Methods of synthesis, fabrication, or derivation of the test, control, or reference substance shall be documented and retained by the sponsor.

- When relevant to the conduct of the study the solubility of each test, control, or reference substance shall be determined by the sponsor before the experimental start date. The stability of the test, control, or reference substance shall be determined by the sponsor before the experimental start date or concomitantly according to written standard operating procedures, which provide for periodic analysis of each batch.

The test substance will be tested as supplied by the sponsor unless directed otherwise. All operations performed on the test substance such as dilution or

specialized storage conditions must be specified by the sponsor before initiation of testing.

The sponsor assures MicroBioTest testing facility management that the test substance has been appropriately tested for identity, strength, purity, stability, and uniformity as applicable.

MicroBioTest will retain all unused test substances after completion of the test for one year, and will only discard them with client permission in a manner that meets the approval of the safety officer.

B. Materials supplied by MicroBioTest, including, but not limited to:

1. Challenge microorganism (requested by the Sponsor of the study): *Mycoplasma orale*, ATCC 23714

   Note: an organic load of 5% serum in the inocula will be used.

2. Laboratory equipment and supplies.

3. Media and reagents:

   a. Suitable broth media: Mycoplasma medium (MM) [ATCC Medium 243]
   b. Suitable agar media: Mycoplasma medium with agar (MMA)
   c. Neutralizer: Recovery Broth with Neutralizer(s)
   d. Phosphate Buffered Saline (PBS)
   e. Letheen Broth (LB)
   f. Heat-inactivated Fetal Bovine Serum

3. Laboratory equipment and supplies, including glass microscope slides (1" x 3" with a 1" x 1" surface for contamination and treatment)

TEST SYSTEM IDENTIFICATION:

All test and control tube racks will be labeled with microorganism, test substance (if applicable) and project number prior to initiation of the study and during incubation. Petri dishes will be labeled with microorganism prior to initiation of the study and microorganism and project number during incubation.

EXPERIMENTAL DESIGN:

A. Inocula preparation:

Mycoplasma from stock cultures will be transferred into the appropriate growth media and incubated for 48±2 hours at 36±1C under anaerobic conditions. A minimum of three consecutive transfers will be performed (but no more than 30). Tubes of 5-mL MM will be inoculated with one loopful of inoculum per tube and incubated at 36±1C under anaerobic conditions. After 48±2 hours, cultures will be used for contaminating the carriers.

On the day of the test, 72±2 hour cultures will be pooled in a sterile container and mixed (when multiple culture tubes are used), and adjusted as necessary to assure that the minimum carrier count range will be achieved using PBS.

After the necessary preparation procedures on the day of the test, the inoculum will be agitated on a Vortex-type mixer for 3-4 seconds (inoculum will be transferred into sterile tubes prior to vortex-mixing, if pooled), allowed to sit for ten minutes and then decanted into a sterile tube(s).

Heat-inactivated Fetal Bovine Serum will be added to each prepared culture to yield an organic soil final concentration of 5%. The addition of an organic challenge will provide a worse case condition for the validation.

B. Carrier preparation and inoculation:

The new carriers will be visually screened and discarded if visibly damaged (scratched, chipped or nicked). The carriers will be rinsed with 95% ethanol followed by a rinse with deionized water to remove oil and film on the slides. The carriers will be sterilized by placing in evaporating dishes matted with two pieces of filter paper, heating them in a hot air oven for two hours at 180C, cooling and storing them at room temperature until use.

Using a positive displacement pipet, a 0.01 mL (10 µL) aliquot of each culture will be transferred onto a one-square inch area on the sterile carriers (in Petri dishes) and immediately spread uniformly over the entire area with a sterile glass rod. Each dish will be covered promptly and the operation will be repeated for the rest of the carriers. Carriers will be dried for 30-40 minutes at 36±1C. The humidity level of the incubator during the drying phase required for the inoculated carriers will be monitored and reported. Inoculated carriers will be used within two hours of drying.

C. Test substance preparation:

The test substance will be prepared and applied exactly as directed by the sponsor of the study. If mixing of components or dilution is required, the prepared test substance will be used within three hours for testing. The test substance will be allowed to equilibrate to room temperature for a minimum of 10 minutes.

D. Test:

Note: The temperature and humidity level of the laboratory during the test phase will be monitored and reported.

Ten carriers per lot will be sprayed in a horizontal position until thoroughly wet from a distance of 6" – 8". Each carrier will be held in a horizontal position for the exposure time as specified by the sponsor. After the contact period, the excess liquid will be allowed to drain from the carrier without touching the Petri dish or filter paper. The carriers will be transferred to tubes containing 20 mL of Neutralizer using sterile forceps within the ±5 sec (or ±3 sec) time limit and shaken thoroughly. For test substances with ≤1 minute contact time, the transfer will be made within ±3 seconds.

All plates will be incubated for 48±2 hours at 36±1°C under anaerobic conditions.. All observations will be recorded as growth or no growth.

If required due to the opacity of the Neutralizer, all test and control tubes will be streaked onto suitable agar media and the plates will be incubated for 48±2 hours at 36±1C under anaerobic conditions. The presence or absence of growth will be determined based on the streaks.

E. Controls:

1. Sterility control:

One sterile carrier will be added to a tube of Neutralizer and incubated with the test in order to demonstrate the sterility of the media used in the study.

2. Viability control:

Two inoculated carriers will be independently transferred into tubes of Neutralizer and incubated with the test to serve as comparison for the test cultures.

3. Neutralizer effectiveness:

For each lot of test substance, two sterile carriers will be exposed to the disinfectant for the required contact time, and then transferred into individual tubes of Neutralizer. To each tube, fewer than 100 colony forming units (CFU) of the challenge microorganism will be added and the count of the bacteria inoculated into these tubes will be confirmed by plating in duplicate using suitable agar media. The tubes and plates will be incubated with the test.

4. Carrier counts:

The average CFU per carrier will be determined using three inoculated carriers immediately after the conclusion of the processing of the test replicates.

Dried inoculated carriers will be placed individually into tubes containing 20 mL LB. The tubes will be immediately vortexed for 120±5 seconds. After vortexing, serial ten-fold dilutions of each suspension will be performed in PBDW blanks. Duplicate one mL aliquots from selected dilutions will be plated using suitable agar media. Diluting and plating will be completed within 2 hours after vortex-mixing. All plates will be incubated with the test and the average CFU/carrier determined.

5. Bacteriostasis control:

If, after two days incubation, no growth is observed in any of the test tubes, at least 20% of the test tubes will be streaked onto suitable agar media and incubated for 48±2 hours at 36±1C under anaerobic conditions. No growth on these plates will negate bacteriostasis as the cause for lack of growth in the test tubes.

If streaking is required due to the opacity of the Neutralizer (See Section D), the test streaks will serve as the evaluation of bacteriostasis.

6. Confirmation of challenge microorganism:

All of the viability controls and at least 20% of the test tubes exhibiting growth will be streaked onto suitable agar media plates. All plates will be incubated for 48±2 hours at 36±1C under anaerobic conditions. Colony morphology we be observed to confirm growth of the challenge microorganism.

If streaking is required due to the opacity of the Neutralizer (See Section D), Gram stains will be performed from the viability control streaks, as well as any positive test streaks, in order to confirm growth of the challenge microorganism.

PRODUCT EVALUATION CRITERIA:

According to the EPA, the test substance passes the test if no visible growth is observed any of the test tubes (0/10) and the controls meet their stipulated criteria. There is no statistical method proposed for this protocol.

TEST ACCEPTANCE CRITERIA:

The test will be acceptable for evaluation of the test results if the criteria listed below are satisfied. The study director may consider other causes that may affect test reliability and acceptance.

- The geometric mean of the carrier counts must be at least 1.0×10^4 CFU/carrier.
- The $\log_{10}$ density (LD) for each carrier will be determined based on the following:
  - Dilutions yielding counts up to 300 CFU will be used.
  - Plate counts of 0 will be included in the calculations.
  - The CFU/mL (of broth) will be calculated:

$$\text{CFU/mL} = \frac{(\text{avg.CFU for } 10^{-x}) + (\text{avg.CFU for } 10^{-y}) + (\text{avg.CFU for } 10^{-z})}{10^{-x} + 10^{-y} + 10^{-z}}$$

  - The CFU/carrier will be calculated by multiplying the CFU/mL by the volume of broth into which the bacteria were harvested from the carrier by vortex-mixing (20 mL).
  - The LD for each carrier will be calculated by taking the Log_{10} of the density (per carrier).
- The recovery broth with neutralizers must be proven effective
- The sterility control must be negative for growth
- The viability control must be positive for growth
- The purity of the challenge microorganism must be confirmed based on the procedures employed for confirmation

DATA PRESENTATION:

The final report will include the following information:

- The number of positive carriers.
- The average colony-forming units per carrier.
- The results of all controls.

PERSONNEL AND TESTING FACILITIES:

A study director will be assigned before initiation of the test. Resumes for technical personnel are maintained and are available on request. This study will be conducted at MicroBioTest, 105 Carpenter Drive, Sterling, VA 20164.

CONFIDENTIALITY:

All data generated at MicroBioTest are held in strictest confidence and are available only to the sponsor and the sponsor designated authorities (if applicable). In turn, no reference to MicroBioTest's promotion of the evaluated test articles may be made public by the sponsor.

REPORT FORMAT:

MicroBioTest employs a standard report format for each test design. Each final report provides the following information:

- Sponsor identification and test substance identification
- Type of test and project number
- Dates of study initiation and completion
- Interpretation of results and conclusions
- Test results
- Methods and evaluation criteria
- Signed Quality Assurance and Compliance Statements (if applicable)

REGULATORY COMPLIANCE AND QUALITY ASSURANCE (applicable to GLP studies only)

This study will be performed in compliance with the US Environmental Protection Agency's Good Laboratory Practices regulations, 40 CFR 160. Note: information on the identity, strength, purity, stability, uniformity, and dose solution analysis of the test substance resides with the sponsor of the study unless otherwise stated.

The Quality Assurance Unit of MicroBioTest will inspect the conduct of the study for GLP compliance. The dates of the inspections and the dates that findings are reported to the study management and study director will be included in the final report.

RECORDS TO BE MAINTAINED:

All raw data, protocol, protocol modifications, test substance records, final report, and correspondence between MicroBioTest and the sponsor will be stored in the archives at MicroBioTest, 105 Carpenter Drive, Sterling, Virginia 20164 or in a controlled facility off site.

All changes or revisions to this approved protocol will be documented, signed by the study director, dated and maintained with this protocol. The sponsor will be notified of any change, resolution, and impact on the study as soon as practical.

The proposed experimental start and termination dates; additional information about the test substance; challenge microorganism used; media and reagent identification; and the type of neutralizers employed in the test will be addressed in a project sheet issued separately for each study. The date the study director signs the protocol will be the initiation date. All project sheets will be forwarded to the study sponsor.

MISCELLANEOUS INFORMATION:

The following information is to be completed by sponsor before initiation of study:

A. Name and address:

Virox Technologies, Inc.
2770 Coventry Road
Oakville, Ontario
L6H 6R1

B. Test substance information:

Test substance name	Oxyteam	
Active ingredient(s)	Hydrogen Peroxide	
Lot No.	Lot No. 1	Lot No. 2
	Lot# 12296	Lot # 12297
Lower Certified Limit (LCL)	☑ yes ☐ No	☐ Not Applicable
Treatment	Spray until thoroughly wet from a distance of 6" - 8"	

06/07/16 JL

Dilution: 1:64 Diluent: AOAC Synthetic Hard Water: 200ppm

C. Test Conditions:

Contact time	10 ~~seconds~~ minutes
Contact temperature	Ambient Room Temperature (20±1C)

D. Organic load – serum (HI FBS) added to achieve 5% in the inoculum: ☑ yes ☐ no

E. Precautions/storage conditions: MSDS and/or C of A provided: ☑ yes ☐ no

REPORT HANDLING: The sponsor intends to submit this information to: US EPA

STUDY CONDUCT: GLP

PROTOCOL APPROVAL:

Sponsor Signature: _____ Date: 06/10/16

Printed Name: _____

Study Director Signature: _____ Date: 06/28/16

Printed Name: MUHAMMAD HAMID BAGHIR